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Moraxella species as potential sources of MCR-like polymyxin-resistance determinants

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Plasmid-mediated resistance to polymyxins mediated by the MCR-1/-2 determinants has been recently reported in *Enterobacteriaceae* worldwide. Using PCR-based and cloning strategies, a series of *Moraxella* species were screened for *mcr*-like genes. *Moraxella* spp. that are mainly animal pathogens but may also be human pathogens were identified as potential reservoirs of *mcr*-like genes.

Resistance to polymyxins in *Enterobacteriaceae* results mostly from chromosomal mutations in genes involved in modification of the lipopolysaccharide (LPS) (1, 2). In particular, it has been shown that mutations, truncations, or insertions into genes encoding LPS-modifying enzymes may be responsible for acquired resistance to polymyxins in *Enterobacteriaceae* (1, 3, 4), *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (1, 5). However, the plasmid-mediated polymyxin resistance determinant MCR-1 has been reported recently from *Escherichia coli* and *Klebsiella pneumoniae* isolates recovered from humans and animals in China (6). Then the *mcr-1* gene has been reported worldwide in other enterobacterial genus including *Enterobacter*, *Salmonella*, and *Shigella* (7-11). It has been identified in Enterobacteriaceae isolated from various animal species, including cattle, chicken, and pigs (12-14), and from river samples and vegetables (15).

MCR-1 is a 541 amino-acid long phosphoethanolamine transferase that adds phosphoethanolamine to the lipid A moiety of the LPS, leading to a more cationic LPS structure, and consequently to resistance to polymyxins (6). Recently, the MCR-

2 protein (538 amino-acid long, 81% amino-acid identity with MCR-1) has been identified in Belgium from colistin-resistant *E. coli* (16). Additionally, the MCR-1.2 and MCR-1.3 variants (both exhibiting single amino acid substitutions compared to MCR-1) have been identified in Italy and China, respectively (17, 18). Several plasmid types carrying the *mcr-1* gene have been identified, belonging either to the IncI2, IncHI2, or IncX4 incompatibility groups (8, 10, 12). In addition, both the *mcr-1.2* and *mcr-2* genes have also been identified onto IncX4 plasmid scaffolds (16, 17).

It is highly suggested that animals treated with polymyxins might represent a significant reservoir of polymyxin-resistant Gram-negative isolates, and in particular of MCR-producing isolates (19). In fact, polymyxins are heavily used in veterinary medicine with Spain and Italy being the country with the highest consomption for food-producing animals in Europe (20). The frequent identification of MCR-1-producing isolates from animals further enhances the hypothesis that the animal world might be the hot spot of this resistance determinant.

In the initial published works reporting MCR-1 and MCR-2, respectively (6, 16), a certain degree of sequence identity between the *mcr-1* and *mcr-2* genes and

some intrinsic chromosomal genes from *Moraxella* species was highlighted. Our hypothesis was that those latter might be candidates to be considered as potential progenitors of different *mcr*-like genes.

Not only sequence analyses of *mcr*-like genes was considered, but also the following features; first, the *mcr-1* gene is very often associated to the insertion sequence (IS) IS*Apl1* that might be responsible for its mobilization (8). That IS has been originally identified in *Actinobacillus pleuropneumoniae* belonging to the *Pasteurellaceae* family (21). That species is responsible for porcine pleuropneumonia and is widely found in pigs (22). Second, many *mcr-1*-positive isolates identified co-harbor resistance determinants corresponding to specific antibiotics used in veterinary medicine, such as the *floR* gene encoding resistance to florfenicol (19). Many published studies showed the wide dissemination of *mcr-1*-positive *E. coli* isolates mostly in animals (14, 23). Finally, the IncX4-type plasmids that are often identified as supports of the *mcr-1* gene have been identified in enterobacterial strains recovered from pigs, regardless of the presence of *mcr-1* (24, 25).

Our focus was therefore made on species belonging to the *Moraxella* genus. *Moraxella* species are Gram-negative bacteria being commensals or pathogens of animals (cattle, sheep, cats, dogs, rabbits and pigs), with *Moraxella catarrhalis* being a mucosal commensal or pathogen for humans (26). In-silico analysis over Genbank databases revealed that a gene encoding a putative MCR-like protein was located onto the chromosome of the *M. catarrhalis* genome, sharing 59 and 60% amino acid identity with MCR-1 and MCR-2, respectively.

Genomic DNAs were extracted from a series of strains belonging to different *Moraxella* species, corresponding to *Moraxella lincolnii* CIP103462, *Moraxella bovoculi* CIP109558T, *Moraxella porci* CIP110214T, *Moraxella lacunata* CIP108000, *Moraxella saccharolytica* CIP57.36 and CIP68.37, *Moraxella bovis* CIP70.40T and CIP103741, *Moraxella equi* CIP82.26T, *Moraxella canis* CIP103801T, *Moraxella caprae* CIP104714T, *Moraxella boevrei* CIP104716T, *Moraxella nonliquefaciens* CIP100617, *Moraxella laennata* CIP102083, and *Moraxella osloensis* CIP100025. PCR-based experiments were performed by using specific and internal primers for both the *mcr-1* and *mcr-2* genes, namely MCR-1+2-

For (5'-TATCGCTATGTGCTAAAGCC-3') and MCR-1+2-Rev (5'-TCTTGGTATTTGGCGGTATC-3'). The annealing temperature was decreased to 50°C in order to allow the amplification of DNA targets that might be not fully homologous to the *mcr* genes. For those PCR giving positive results, only internal amplifications of the *mcr*-like genes were obtained. In order to gain knowledge about the entire *mcr*-like genes, an inverse PCR strategy was used, as reported (27). Briefly, outward primers were designed from the internal sequenced fragment in order to amplify adjacent DNA sequences. Templates corresponded to genomic DNAs of the strains that had been previously digested with different restriction enzymes (BamHI, HindIII, EcoRI), and self-circularized using DNA T4 ligase.

PCR assays with internal *mcr-1*-specific genes gave positive results for three strains, corresponding to *M. lincolnii, M. porci*, and *M. osloensis*. Subsequently the entire *mcr*-like genes were identified using the inverse-PCR strategy. The corresponding proteins respectively named MCR-LIN for *M. lincolnii*, MCR-POR for *M. porci*, and MCR-OSL for *M. osloensis*, shared significant amino acid identities with MCR-1 and MCR-2, ranging from 59% to 64%, respectively (Table 1).

Noteworthy, the most closely-related variant compared to MCR-1 was identified in *M. porci*, while the most closely-related variant of MCR-2 was identified in *M. osloensis.* Those identities were also calculated for the corresponding genes at the nucleotide levels and further confirmed the significant degree of similarity with *mcr*-like genes sharing ca. 70% identity with the *mcr-1* and *mcr-2* genes (Table 2).

Interestingly, when considering the recently determined structure of the catalytic domain of MCR-1 (28), all MCR-like proteins identified in this study possess the six cysteine residues forming the three disulfide bridges of the catalytic domain (Figure 2). The threonine 285 residue shown to be the catalytic nucleophile was also conserved in all those proteins (28). A phylogenetic tree was elaborated with nucleotide sequences of the genes encoding the different MCR-like enzymes along with other putative phosphoethanolamine transferase proteins. Interestingly, MCR-1 and MCR-2 significantly clustered with those newly-identified MCR-like determinants from *Moraxella* spp. (Figure 2, panel A). Noteworthy, the phosphoethanolamine transferase from *Enhydrobacter aerosaccus* also clustered in that group. This result is in accordance with the recent taxonomic reclassification of

that species into the family Moraxellaceae (29). In parallel, the phylogenetic tree elaborated with sequences of the 16S RNA gene sequences of those different bacterial species confirmed that feature (Figure 2, panel B).

When analyzing the nucleotide sequences located dowstream of *mcr*-like genes, the significant identity between the mobilized fragment encompassing either *mcr-1* or *mcr-2* and those identified in the chromosome of the *Moraxella* species were evidenced. Indeed, homologues of the 240 amino-acid long open reading frame previously identified downstream of *mcr-1* were also identified downstream of the *mcr*-like genes, sharing ca. 40-50% amino acid identity (Table 2). This protein encodes a putative PAP2 membrane-associated lipid phosphatase (16).

A PCR-based screening of the different *Moraxella* strains for the presence of the replicase gene of IncX4 plasmids was performed with primers X4-Fw and X4-Rv, as published (8). Interestingly, a positive signal was obtained using the genomic DNA of *M. lacunata*, that was further confirmed to be 99% identical to the IncX4 replicase gene identified in association with the *mcr-1* gene, therefore showing that *M. lacunata* may be also a reservoir of IncX4-type plasmids.

Since the *mcr-1* gene is most often associated to the IS*Apl1* element, a PCR specific for that IS element was performed with all *Moraxella* strains. A positive signal was found with the *M. porcii* strain, and sequencing confirmed a perfect identity with IS*Apl1*. Noticeably, IS*Apl1* was not located upstream of the *mcr-por* gene, by contrast to what is observed for both *mcr-1* and *mcr-2* genes. This result highlights that some *Moraxella* species may possess in their genome both the putative resistance gene along with the genetic tool likely involved in their mobilization, therefore reinforcing the possibility of occurrence of such mobilization process. Noteworthy, we recently demonstrated that IS*Apl1* elements could mobilize the *mcr-1* gene by transposition (manuscript in revision).

The different *mcr*-like genes were cloned in the same L-arabinose inducible vector pBADb (30). Recombinant plasmids were expressed into *E. coli* TOP10 by adding 1% L-arabinose and MIC values of colistin were determined by broth dilution methods, as recommended by CLSI (31). Production of MCR-LIN and MCR-POR conferred 8- or 16-fold increased MIC values of colistin in *E. coli* (from 0.03 to 0.5 or 1 µg/ml), and higher (64 fold) was the MIC increase conferred by MCR-OSL (4

µg/ml). Nonetheless, using the exact same background, the highest MIC value of colistin was achieved with MCR-1, thus highlighting that MCR-1 was the most efficient enzyme to confer acquired resistance to colistin in *E. coli*. Noteworthy, both *M. lincolnii* and *M. porci* showed high MIC values of colistin (64 µg/ml).

This report indicates that *Moraxella* constitute a reservoir of *mcr*-like genes that might be mobilized from their original host to become acquired resistant determinants in clinically-significant species, as previously shown for other resistance genes, with *Kluyvera* spp. being the sources of CTX-M-type extended-spectrum ß-lactamase genes (32, 33), *Citrobacter freundii*, *Morganella morganii* and *Hafnia alvei* of AmpC-type ß-lactamase genes (34, 35, 36), *Shewanella* spp. of OXA-48 and OXA-181 carbapenemase genes (37, 38), and *Shewanella algae* of the plasmid-mediated quinolone resistance determinants QnrA (39).

It is tempting to speculate that the heavy usage of polymyxins in animals may be a selective factor for mobilizing those naturally-occurring MCR resistance determinants from *Moraxella*-like species to Enterobacteriaceae. In addition, we showed here that all genetic features requested for mobilization of the *mcr-like* genes

(bacterial progenitor, genetic tools, and selective presure) are present in *Moraxella* that may be providers of further clinically-relevant and plasmid-mediated MCR-like determinants in the future. The exact species acting as progenitors of the MCR-1 and MCR-2 encoding genes remains however to be determined.

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Legend of Figure

Figure 1. Sequence comparison of plasmid-mediated MCR-like determinants. The plasmid-mediated MCR-1 and MCR-2 determinants are from (6, 16). MCR-LIN is from *M. lincolnii*, MCR-CAT from *M. catarrhalis,* MCR-OSL from *M. osloensis*, and MCR-POR from *M. porci.* Dots are for identical amino acid residues. The conserved cysteine residues forming the three disulfide bridges of the MCR-1 catalytic domain are shaded (28). The threonine 285 that was shown to be the catalytic nucleophile in MCR-1 (28) is also conserved among all those MCR proteins, and bracketed.

Figure 2. Phylogenetic tree obtained for a series of phosphoethanolamine transferase encoding genes (panel A) and 16S RNA genes (panel B) by the distance method using Neighbor-Joining algorithm (SeaView version 4 software) (40). Branch lengths are drawn to scale and are proportional to the number of amino acid changes with 200 bootstrap replications. The distance along the vertical axis has no significance.

Table 1. Percentages of identity at the amino acid (and nucleotide) levels between plasmid-mediated MCR-1/MCR-2 polymyxin resistance determinants and those identified in the different *Moraxella* species, namely *M. porcii*, *M. osloensis*, *M. lincolnii*, and *M. catarrhalis*.

	MCR-1	MCR-2	MCR-POR	MCR-OSL	MCR-LIN
MCR-2	81 (79)				
MCR-POR	63 (70)	62 (70)			
MCR-OSL	63 (68)	64 (68)	62 (65)		
MCR-LIN	59 (67)	60 (70)	59 (67)	59 (66)	
MCR-CAT	59 (67)	60 (69)	59 (67)	59 (66)	99 (98)

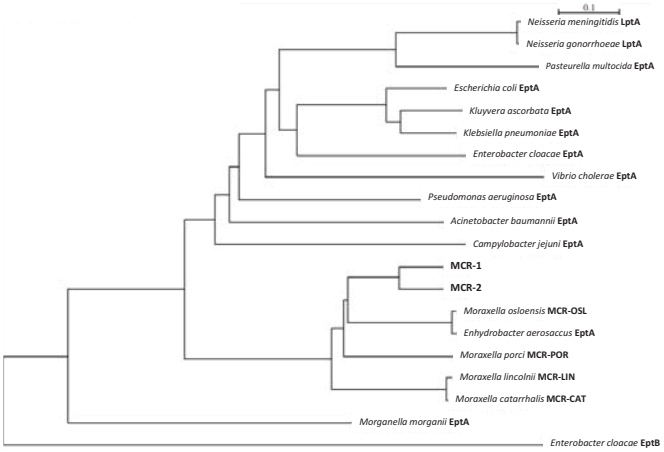
Table 2. Analysis of the putative open reading frame (ORF) regions located downstream of the *mcr*-like genes

Gene	ORF length (amino acids)	Distance from *mcr* stop codon (nucleotides)	Identity (amino acids) of ORF_{MCR-1} (coverage)[a]
mcr-1	240	71	-
mcr-2	82[a]	93	51% (97%)[c]
mcr_{OSL}	236	90	44% (90%)
mcr_{CAT}	251	45	38% (89%)
mcr_{LIN}	245	50	39% (91%)
mcr_{POR}	216	48	43% (86%)

[a]The coverage represents the percentage of the query sequence that overlaps the subject sequence (i.e ORF_{MCR-1}). [b]Putative ORF of MCR-2 is truncated by a copy of IS*1595*. [c]Coverage was calculated from the alignment of the truncated ORF_{MCR-2} and the first 84 amino acids of ORF_{MCR-1}. The mcr_{OSL}, mcr_{CAT}, mcr_{LIN}, and mcr_{POR} genes are respectively from *M. osloensis*, *M. catarrhalis*, *M. lincolnii*, and *M. porcii*.

Figure 2

(A)



(B)